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A novel PI3K inhibitor iMDK suppresses non-small cell lung Cancer cooperatively with A MEK inhibitor

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Abstract

The PI3K–AKT pathway is expected to be a therapeutic target for non-small cell lung cancer (NSCLC) treatment. We previously reported that a novel PI3K inhibitor iMDK suppressed NSCLC cells *in vitro* and *in vivo* without harming normal cells and mice. Unexpectedly, iMDK activated the MAPK pathway, including ERK, in the NSCLC cells. Since iMDK did not eradicate such NSCLC cells completely, it is possible that the activated MAPK pathway confers resistance to the NSCLC cells against cell death induced by iMDK. In the present study, we assessed whether suppressing of iMDK-mediated activation of the MAPK pathway would enhance anti-tumorigenic activity of iMDK. PD0325901, a MAPK inhibitor, suppressed the MAPK pathway induced by iMDK and cooperatively inhibited cell viability and colony formation of NSCLC cells by inducing apoptosis *in vitro*. HUVEC tube formation, representing angiogenic processes *in vitro*, was also cooperatively inhibited by the combinatorial treatment of iMDK and PD0325901. The combinatorial treatment of iMDK with PD0325901 cooperatively suppressed tumor growth

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and tumor-associated angiogenesis in a lung cancer xenograft model *in vivo*. Here, we demonstrate a novel treatment strategy using iMDK and PD0325901 to eradicate NSCLC.

Keywords

PI3K; MEK; NSCLC

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide [1]. Recent discoveries of molecularly targeted therapies, including erlotinib/gefitinib for *EGFR*-mutant NSCLC [2,3] and crizotinib/ceritinib for *ALK*-fusion NSCLC [4,5] have demonstrated that such therapies using small molecule drugs extend survival of NSCLC patients significantly, compared to conventional chemotherapy [6,7]. In addition to *EGFR*-mutant or *ALK*-fusion NSCLCs, progression for *RET*-fusion and *ROS1*-fusion NSCLCs mortality are also considered to be favorable since they can be targeted by small molecules, such as crizotinib [7,8]. A drawback of current therapy for NSCLC is that there is no effective treatment for *KRAS*-mutant NSCLC; the most common type in Caucasians and the second most common type in Asians [9], and squamous NSCLC [10]. Molecularly targeted therapies for *KRAS*-mutant NSCLC have been tested [11,12]. For example, inhibition of MEK (a MAPKK; downstream of *KRAS*) by selumetinib, a MEK inhibitor, in addition to docetaxel (a chemotherapeutic drug) extended survival of *KRAS*-mutant NSCLC patients by 4 months compared to docetaxel alone. However, selumetinib plus docetaxel caused more adverse events than docetaxel alone [13], indicating that further improvements in this therapeutic strategy are needed to extend survival and reduce the adverse events associated with *KRAS*-mutant NSCLC.

In addition to the *KRAS*–MAPK pathway, the *KRAS*–PI3K–AKT pathway is considered to contribute to *KRAS* lung tumorigenesis [14]. While not mutually exclusive, these pathways often interact with each other [15]. For example, a PI3K inhibitor PI-103 inhibited the PI3K pathway while activating the MAPK pathway [16], suggesting a potential mechanism for tumor survival by the compensatory activation of an alternative tumorigenic pathway. We recently reported a novel PI3K inhibitor iMDK, which was originally identified as a small molecule inhibiting the growth factor MDK (also known as midkine or MK) [17]. While its direct targets are unknown, iMDK inhibited phosphorylation of PI3K and AKT, indicating that iMDK serves as a PI3K inhibitor [17]. Similarly to PI-103 [16], iMDK activated phosphorylation of ERK and P38MAPK while inhibiting the PI3K pathway [17]. Since the combined inhibition of the PI3K and MAPK pathways by PI-103 and a MAPK inhibitor PD0325901 enhanced cell killing of NSCLC [16], in the present study, we tested whether the novel PI3K inhibitor iMDK further enhanced cell death of NSCLC in the presence of PD0325901. Combined treatment of iMDK and PD0325901 significantly suppressed tumor growth of NSCLC, including *KRAS*-mutant NSCLC and squamous NSCLC. The combinatorial treatment also inhibited endothelial cell-mediated angiogenesis, a requirement for tumor progression *in vitro* and *in vivo*. Here, we demonstrate a novel strategy to treat

NSCLC by simultaneously targeting both PI3K and MAPK pathways by iMDK and PD0325901.

Materials and methods

Reagents

3-[2-(4-fluorobenzyl)imidazo[2,1-b][1,3]thiazol-6-yl]-2H-chromen-2-one (here after termed iMDK) purchased from ChemDiv (San Diego, CA) and MEK inhibitor PD0325901 obtained from Selleck Chemicals (Houston, TX) were dissolved in DMSO.

Cell lines and culture conditions

H441 (lung adenocarcinoma; *KRAS*^{G12V}), H2009 (non-small cell carcinoma; *KRAS*^{G12A}), A549 (lung carcinoma; *KRAS*^{G12S}) and H520 (lung squamous cell carcinoma; *KRAS*^{WT}) were obtained from the American Type Culture Collection (Manassas, VA) and grown in high glucose Dulbecco's modified Eagle medium (H441 and A549) or RPMI 1640 (H2009 and H520) supplemented with 10% heat-inactivated fetal bovine serum. Cell information was obtained from COSMIC (<http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/>). Human umbilical vein endothelial cells (HUVECs) purchased from Life Technologies (Grand Island, NY) were grown in HUVEC growth medium (MCDB-104 medium supplemented with 10 ng/ml EGF, 100 µg/ml heparin, 100 ng/ml endothelial cell growth factor and 10% heat-inactivated fetal bovine serum). All cell lines were cultured in 10% CO₂ at 37 °C.

Immunoblot analysis

Cells were lysed in ice-cold M-PER lysis buffer purchased from Thermo Fisher Scientific (Rockford, IL). Cell lysates were clarified by centrifugation (20 min at 15,000g at 4 °C) and protein concentration determined using the BCA protein assay (Thermo Fisher Scientific). Equal amounts of protein were separated on an SDS-PAGE gel. The gel was electrophoretically transferred to a Hybond PVDF transfer membrane (GE. Healthcare Ltd., Piscataway, NJ) and incubated with primary and secondary antibodies according to the Supersignal® West Pico chemiluminescence protocol (Pierce, Rockford, IL). Antibody specific for β-actin antibody was obtained from Sigma (St. Louis, MO). Antibody specific for AKT, phosphorylated-AKT (Ser473), ERK, and phosphorylated-ERK (Ser473) were obtained from Cell Signaling Technology (Beverly, MA). Secondary horseradish peroxidase-conjugated antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell viability assay

H441 cells and H2009 cells were plated in 96-well plates at a density of 1.5×10^3 cells and cultured at 37 °C for 24 h. H441 and H2009 cells were treated with or without iMDK (2.5 µM) in the presence or absence of PD0325901 (0.5 µM). H520 cells were treated with or without iMDK (0.125 µM) in the presence or absence of PD0325901 (0.25 µM). A549 cells were treated with or without iMDK (0.25 µM) in the presence or absence of PD0325901 (0.125 µM). Cells were treated for 72 h. Viable cells were assessed by WST-1 assays (Roche Molecular Biochemicals, Laval, Quebec, Canada) according to the manufacturer's protocol.

Colony formation assay

Cells were first plated at a density of 5×10^4 cells for H441 and 1×10^5 cells for H2009 cells per well in 12-well plates 24 h before treatment. H441 cells were treated with iMDK at a concentration of 1 μ M and/or PD0325901 at a concentration of 500 nM for 24 h and then released from the dish by incubation with trypsin/EDTA, counted, plated in triplicate at a density of 5×10^3 cells in six-well plates for 14 days. H2009 cells were treated with iMDK and/or PD0325901 at a concentration of 1 μ M for 24 h and then released from the dish by incubation with trypsin/EDTA, counted, plated in triplicate at a density of 1×10^3 cells in six-well plates for 14 days. The cells were fixed with 100% methanol and allowed to air dry. The cells were then stained with 0.1% crystal violet. Colonies (a group of aggregated cells numbering at least 50) were then counted. The mean number of the control group was arbitrarily set to 100%, and all other numbers were normalized and percentage-specific cytotoxicity compared to colony formation in the control group was calculated.

Flow cytometric analysis for activated caspase-3

H441 cells were plated in 12-well plates at a density of 1×10^5 cells per well 1 day before the treatments. Cells were treated with iMDK (10 nM) or PD0325901 (10 nM) alone, or iMDK (10 nM) in combination with PD0325901 (10 nM). After 72 h, cells were harvested and washed twice with PBS. Cleaved caspase 3 was labeled *in situ* with phycoerythrin (PE)–conjugated anti-activated caspase-3 antibodies purchased from BD Bioscience (San Jose, CAs) and analyzed by FACS Verse (BD Bioscience)[18,19].

TUNEL staining

Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining was performed to detect apoptosis using the DeadEnd colorimetric TUNEL system (Promega, Madison, WI) according to the manufacturer's protocol.

HUVEC tube formation assay

HUVECs were treated with iMDK (10 μ M) and/or PD0325901 (10 μ M) for 5 h and a tube formation assay was performed using an *in vitro* angiogenesis kit (Millipore Corp. Bedford, MA). Tube formation was quantified by measuring the total length of the tube network and the number of branching points using Image J software (NIH, Bethesda) [20,21].

Mouse experiments

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University Graduate School of Medicine and Dentistry (Ethics Committee reference number: OKU-2013104). Human lung cancer xenografts were established in 6-wk-old female BALB/c nude mice (Charles River Laboratories Japan, Kanagawa, Japan) by subcutaneous (s.c.) inoculation of H441 cells ($1 \times 10^6/50 \mu$ l) into the right dorsal flank. The mice were randomly assigned into four groups ($n=8$ per group) 9 days after tumor inoculations. The mice were intraperitoneally injected with 100 μ l solution containing iMDK (9 mg/kg) everyday and/or orally administered PD0325901 (5 mg/kg) in 0.5% [w/v] methylcellulose solution with 0.2% [v/v] polysorbate 80 [Tween 80] five times per week (on days 1, 2, 3, 4, 5, 7, 8, 9, 10, 11). DMSO was intraperitoneally injected and

methylcellulose solution with polysorbate 80 was orally administered in the control group. Tumors were measured every day, and tumor volume was calculated as $a \times b^2 \times 0.5$, where a and b were large and small diameters, respectively. On day 11, all mice were sacrificed and tumors were removed and prepared for histology.

For immunohistochemistry, sections were sequentially depar-affinized through a series of xylene, graded ethanol, and water immersion steps. After being autoclaved in 0.2% citrate buffer for 15 min, sections were incubated with 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. The antibodies for CD31/PECAM-1 were obtained from Abcam. Specimens were incubated overnight at 4 °C with a 1:100 dilution of antibody followed by three washes with TBS. The slides were treated with streptavidin-biotin complex (Envision System labeled polymer, horseradish peroxidase [HRP], Dako, Carpinteria, CA) for 60 min at a dilution of 1:100. Immunoreactions were visualized using a 3,3'-diaminobenzidine (DAB) substrate-chromogen solution (Dako Cytomation LiquidDAB Substrate Chromogen System, Dako) and counterstained with hematoxylin. Sections were immersed in an ethanol and xylene bath and mounted for examination.

Ras activation assay

H441 cells were treated with iMDK (250 nM and 500 nM) or PD0325901 (50 nM and 250 nM) for 6 h and 24 h. Cells were rinsed in PBS before cell lysis. Ras activation was determined using a RasGTPase Chemi ELISA kit, according to manufacturer's protocol (Active Motif, Carlsbad, CA).

Statistical analysis

Significance of group differences was evaluated using ANOVA for multiple comparisons. Statistical significance was defined as $p < 0.05$ (#).

Results

PD0325901 inhibited activation of ERK induced by iMDK treatment in H441 lung adenocarcinoma cells

In the previous study, we demonstrated that iMDK suppressed the PI3K–AKT pathway (12 h after treatment) while inducing the MAPK pathway (48 h after treatment), as detected by the phosphorylation of ERK (MAPK), in H441 lung adenocarcinoma cells that carry a *KRAS* mutation (*KRAS*^{G12V}) [22]. Phosphorylated ERK (p-ERK) was slightly increased less than 1 h after treatment (Supplemental Fig. 1); however, p-ERK was robustly increased 72 h after treatment (Fig. 1A), suggesting that the effect of iMDK on the MAPK pathway in a short time is limited. RAS-GTP levels were not affected by iMDK (less than 24 h; Supplemental Fig. 2). These results suggest that iMDK primarily targets the PI3K pathway but not *KRAS* itself, which in turn influences alternative tumorigenic pathways. Induction of an alternative tumorigenic pathway after suppression of a tumorigenic pathway by therapeutic cancer drugs is considered to be a mechanism by which tumors evade cell death from the molecularly targeted drugs. Thus, a combinatorial treatment using drugs targeting multiple pathways may be required to block tumor cell resistance [15,23–25]. In order to test this concept, we assessed whether the MAPK pathway activated by iMDK is suppressed by

PD0325901, a MEK (MAPKK) inhibitor, in H441 cells. As shown in Fig. 1B, PD0325901 suppressed iMDK-induced phosphorylation of ERK in a dose-dependent fashion. These results indicate that the combinatorial treatment of iMDK with PD0325901 simultaneously inhibits two major *KRAS* tumorigenic pathways in H441 cells.

PD0325901 enhanced iMDK-mediated suppression of cell proliferation and colony formation in H441 cells

Having demonstrated that the combinatorial treatment of iMDK and PD0325901 inhibited both the PI3K pathway and the iMDK-mediated activation of the MAPK pathway in H441 cells carrying *KRAS*^{G12V} (Fig. 1), we assessed whether the combinatorial treatment would inhibit growth of H441 cells as well as other *KRAS* mutated NSCLC cells, including H2009 (non-small cell carcinoma; *KRAS*^{G12A}) and A549 (lung carcinoma; *KRAS*^{G12S}) cells, and *KRAS* wild type H520 (lung squamous cell carcinoma; *KRAS*^{WT}) cells. First, we assessed whether iMDK inhibited the PI3K pathway not only in H441 cells but also other cells, including H2009 and H520 cells. As shown in Supplemental Fig. 3, AKT (downstream of PI3K) activation was inhibited by iMDK, indicating that iMDK inhibits the PI3K pathway in a broad range of NSCLC cells regardless of *KRAS* mutations. Next, we treated the cells with iMDK and/or a MAPK inhibitor PD0325901. As shown in Fig. 2A, PD0325901 further inhibited iMDK-mediated suppression of cell viability in *KRAS* mutated H441 and H2009 cells as well as *KRAS* wild type H520 cells. Though iMDK alone did not inhibit cell viability of A549 cells, the combinatorial treatment of iMDK with PD0325901 significantly inhibited that of A549 cells compared to the single treatment of PD0325901. As shown previously [17], MDK is expressed in H441, H2009 and H520 cells but not in A549 cells. These results indicate that the combinatorial treatment inhibits cell viability of NSCLC cells independently of *KRAS* mutations or MDK expression. In addition to cell viability, colony formation of H441 and H2009 cells were also significantly reduced by the combinatorial treatment compared to either iMDK or PD0325901.

Combinatorial treatment of iMDK with PD0325901 increased apoptosis in H441 cells

In order to analyze the mechanism by which PD0325901 enhances iMDK-mediated cell growth inhibition in the NSCLC cells, we measured the degree of apoptosis after the combinatorial treatment in H441 cells by quantifying activated caspase-3 and TUNEL positive cells, which are markers of apoptosis. As shown in Fig. 3, flow cytometric analysis demonstrated that single use of iMDK or PD0325901 (10 nM) did not induce activation of caspase-3 (Fig. 3A). Combinatorial treatment with iMDK and PD0325901 synergistically increased levels of activated caspase-3. Combinatorial treatment significantly increased the number of TUNEL positive cells compared to either iMDK or PD0325901 alone (Fig. 3B). These results indicate that the inhibition of H441 cell growth by the combinatorial treatment of iMDK with PD0325901 is likely attributed to apoptosis.

iMDK with PD0325901 suppressed tube formation of HUVECs *in vitro*

Endothelial cells contribute to tumor progression by creating blood vessels leading to angiogenesis to fuel tumor cells [26]. We sought to determine whether the combinatorial treatment with iMDK and PD0325901 influences not only NSCLC cells but also

angiogenesis constituted from such endothelial cells. First, using TUNEL staining, we assessed whether the treatment of iMDK, PD0325901 or both in combination would induce cell death in HUVEC (Human Umbilical Vein Endothelial Cells). iMDK, PD0325901 or a combination of the two did not induce cell death in HUVEC *in vitro* (Fig. 4A), indicating that iMDK does not cause apoptosis in normal cells, consistent with our previous report [17]. Next, we assessed whether treatment with iMDK, PD0325901 or both in combination influenced angiogenesis by assessing tube formation in HUVECs, which mimics angiogenesis *in vitro*. Neither iMDK nor PD0325901, when used alone, inhibited angiogenesis as assessed by HUVEC tube formation. In contrast, combinatorial treatment with iMDK and PD0325901 significantly disrupted tube formation 5 h after treatment (Fig. 4B). Protein expression of MDK was not detected in HUVEC cells (data not shown), indicating that the combinatorial treatment disrupts the tube formation independently of MDK. These results indicate that simultaneous inhibition of the PI3K and MAPK pathways suppresses angiogenesis as well as tumor cell growth, both of which are required for tumor growth.

The combinatorial treatment of iMDK with PD0325901 significantly suppressed tumor growth and tumor-associated angiogenesis in lung Cancer xenograft model *in vivo*

In order to determine whether combinatorial treatment of iMDK with PD0325901 cooperatively suppresses lung tumor growth *in vivo*, we treated nude mice carrying xenograft tumors derived from H441 cells with iMDK and/or PD0325901. As shown in Fig. 5A, tumor growth was significantly suppressed after daily intraperitoneal injection of iMDK or oral administration of PD0325901 5 times a week. The combinatorial treatment of iMDK with PD0325901 further inhibited the tumor growth compared to either iMDK or PD0325901 alone. Angiogenesis in the xenografted tumor, which was assessed by the expression of CD31/PECAM-1, was also cooperatively inhibited by the combinatorial treatment of iMDK with PD0325901 compared to either iMDK or PD0325901 (Fig. 5B and C). These results indicate that the combinatorial treatment of iMDK with PD0325901 significantly suppresses tumor growth and tumor-associated angiogenesis of NSCLC *in vivo*.

Discussion

Molecularly targeted therapies are extending survival of some NSCLC patients compared to conventional chemotherapy [7]; however, a therapeutic target for *KRAS*-mutant NSCLC and squamous NSCLC, which are the most common lung cancers, has not been clinically established. A recent clinical trial using a MAPK inhibitor selumetinib, a molecularly targeted drug, in combination with docetaxel (chemotherapeutic [non-molecularly targeted] drug) extended survival by 4 months for patients whose lung adenocarcinomas carry *KRAS* mutations compared to those using docetaxel alone; however, the combinatorial treatment resulted in increased incidence of adverse effects compared to docetaxel only treatment [13]. There is an urgent need for better therapeutic strategies to treat NSCLCs whose molecular targets are not known.

Previously, we reported that iMDK, which suppresses the expression of MDK, inhibited the PI3K pathway and in turn suppressed the growth of *KRAS*-mutant NSCLC cells [17].

Unexpectedly, iMDK also activated the MAPK pathway, which suggests the compensatory activation of an alternative tumorigenic pathway for *KRAS*-mutant NSCLC cell survival. In the present study, killing of *KRAS*-mutant NSCLC cells was enhanced by inhibiting iMDK-mediated activation of the MAPK pathway using PD0325901 in addition to iMDK-mediated suppression of the PI3K pathway. The combinatorial treatment was also effective in reducing tumor growth of *KRAS* wild type H520 squamous NSCLC cells, indicating that the combinatorial treatment can be applicable for different types of NSCLC. iMDK did not reduce body weight or cause toxicity in mice [17], suggesting that the combinatorial use of iMDK with PD0325901 or other MAPK inhibitors, such as selumetinib, might be superior to the combinatorial use of docetaxel and selumetinib to prevent the adverse effects.

Combinatorial therapy with iMDK may also enable treatment using reduced amounts of a MAPK inhibitor. The simultaneous inhibition of both the PI3K and MAPK pathways by small molecule drugs was previously shown to suppress *KRAS*-mutant NSCLC *in vitro* and *in vivo* [15,27]. For example, the combinatorial use of NVP-BEZ235 (a PI3K inhibitor) and ARRY-142886 (a MAPK inhibitor) synergistically reduced *Kras*-mutant lung tumors in a transgenic mouse model [28]. NVP-BEZ235 is currently being tested for safety in clinical trials [29]. While both NVP-BEZ235 and iMDK inhibit the PI3K pathway, the chemical structure of iMDK is distinct from that of NVP-BEZ235, indicating that iMDK may influence the PI3K pathway in a manner distinct from NVP-BEZ235. Clinical development of iMDK or related compounds as a next generation PI3K drug may be useful.

Angiogenesis creates a tumor microenvironment that enhances tumor progression, developing new blood vessels consisting of endothelial cells and thereby supplies nutrients and oxygen to tumor cells [30]. Previously, we demonstrated in a xenograft mouse model using H441 cells that iMDK suppressed expression of CD31/PECAM-1, a marker of angiogenesis [17]. While iMDK suppresses angiogenesis *in vivo*, it remains unknown whether iMDK directly influences growth or activities of endothelial cells. In the present study, we sought to determine whether the combinatorial treatment of iMDK with PD0325901 suppressed angiogenesis. Tube formation assays using HUVEC (endothelial cells) recapitulate the angiogenesis process *in vitro* [31]. The combinatorial treatment of iMDK with PD0325901 suppressed HUVEC tube formation but not HUVEC growth compared to either iMDK or PD0325901 (Fig. 4). The combinatorial treatment also suppressed tumor-associated angiogenesis in the xenograft lung tumor derived from H441 cells compared to those of treatment with a single compound *in vivo* (Fig. 5). These results suggest that the combinatorial treatment suppresses NSCLC growth by not only directly causing apoptosis of NSCLC cells but also by directly disrupting tumor-associated angiogenesis.

In summary, we demonstrated that the combinatorial treatment of a novel PI3K inhibitor iMDK with a MAPK inhibitor PD0325901 cooperatively suppressed the growth of NSCLC cells regardless of *KRAS* mutations or MDK expression. The combinatorial treatment also disrupted *in vitro* and *in vivo* angiogenesis that may influence the tumor-associated environment to limit tumor survival. The strategy using iMDK or related compounds with a MAPK inhibitor may be a promising approach to treat NSCLC whose molecular targets are not identified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.yexcr.2015.03.019>.

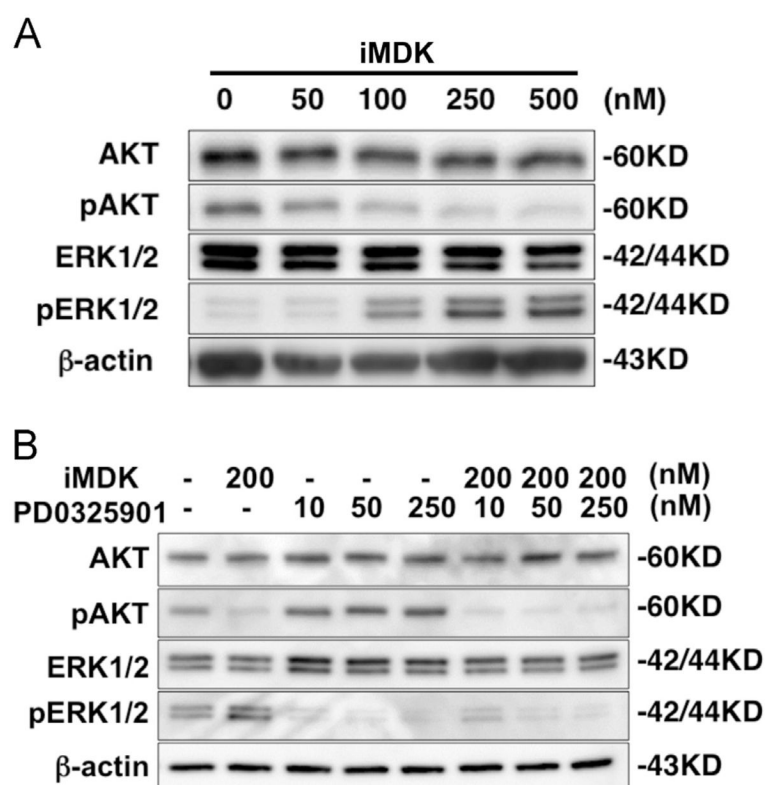


Fig. 1. PD0325901 inhibited ERK1/2 phosphorylation induced by iMDK treatment in H441 lung adenocarcinoma cells

A. iMDK suppressed AKT phosphorylation in a dose-dependent manner (0–500 nM) in H441 lung adenocarcinoma cells after treatment for 72 h. In contrast, ERK1/2 phosphorylation was increased by iMDK as assessed by immunoblot analysis.

B. PD0325901 (10–250 nM) suppressed ERK1/2 phosphorylation induced by iMDK (200 nM) in H441 cells. Conversely, iMDK suppressed AKT phosphorylation induced by PD0325901. H441 cells were treated with iMDK in the presence or absence of PD0325901 for 72 h.

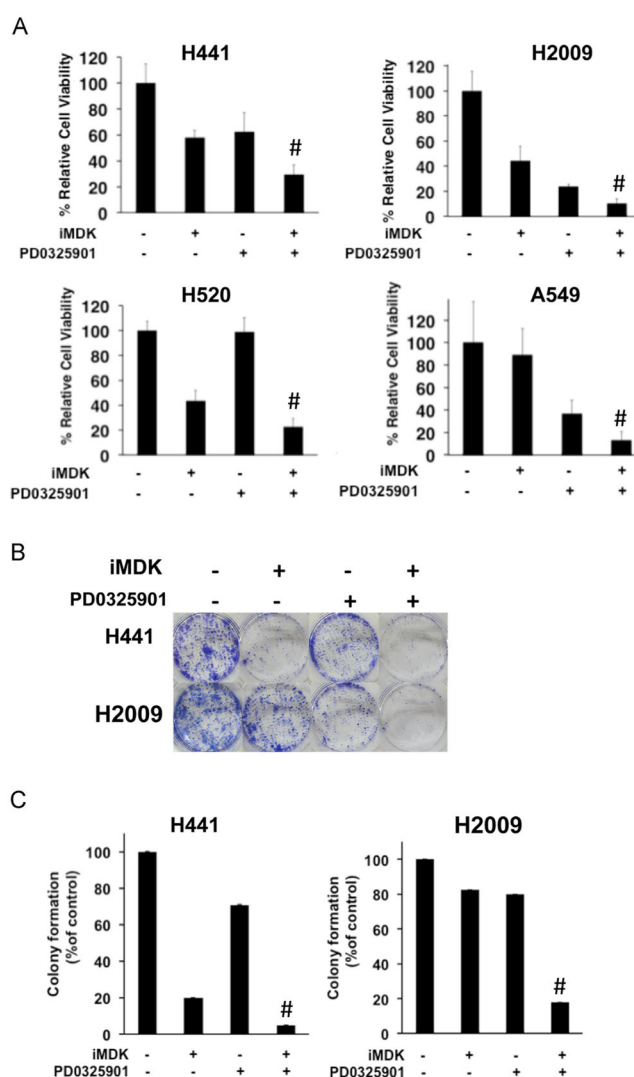


Fig. 2. Combination of iMDK and PD0320901 cooperatively suppressed cell viability and colony formation of NSCLC cells

A. PD0325901 enhanced growth inhibition in H441, H2009 and H520 and A549 NSCLC cells after iMDK treatment. Treatment conditions were described in Materials and Methods. Cell viability was assessed by the WST-1 assay 72 h after exposure to iMDK. Data are presented as % growth inhibition as described in *Methods*. Statistical significance was defined as $p < 0.05$ (#).

B. Colony formation of H441 and H2009 cells treated with iMDK (1 μ M) and/or PD0325901 (500 nM for H441 cells, 1 μ M for H2009 cells). Fourteen days after the treatment, cells were fixed and stained with crystal violet. Representative images of experiments performed in triplicate are shown.

C. Mean colony number was derived from quantitation of triplicate dishes for each treatment and arbitrarily set to 100%. Data are shown relative to controls. Statistical significance was defined as $p < 0.05$ (#).

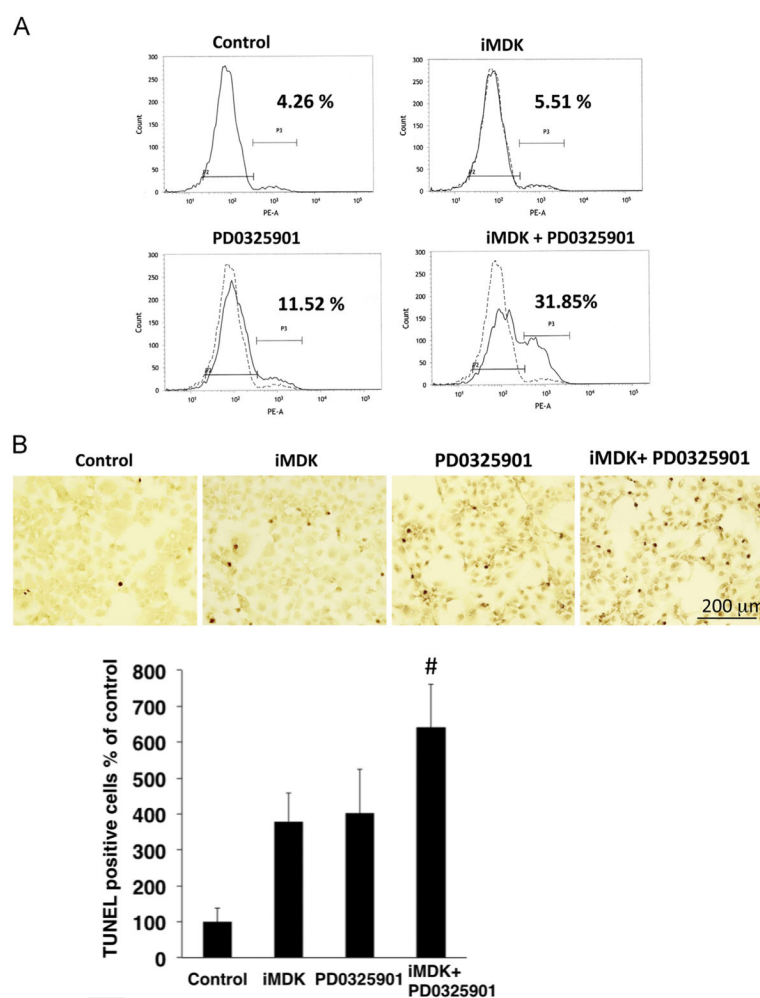


Fig. 3. PD0325901 enhanced apoptosis induced by iMDK in H441 lung adenocarcinoma cells

A. H441 lung adenocarcinoma cells were fixed and stained with anti-activated caspase 3 antibodies 72 h after iMDK (10 nM) and/or PD0325901 (10 nM) and analyzed by flow cytometry. The percentage of cells staining for active caspase-3 is indicated.

B. PD0325901 enhanced apoptosis induced by iMDK in H441 cells. Cells were treated for 72 h at a concentration of 10 nM iMDK. TUNEL staining was performed as described in *Methods*. Statistical significance was defined as $p < 0.05$ (#).

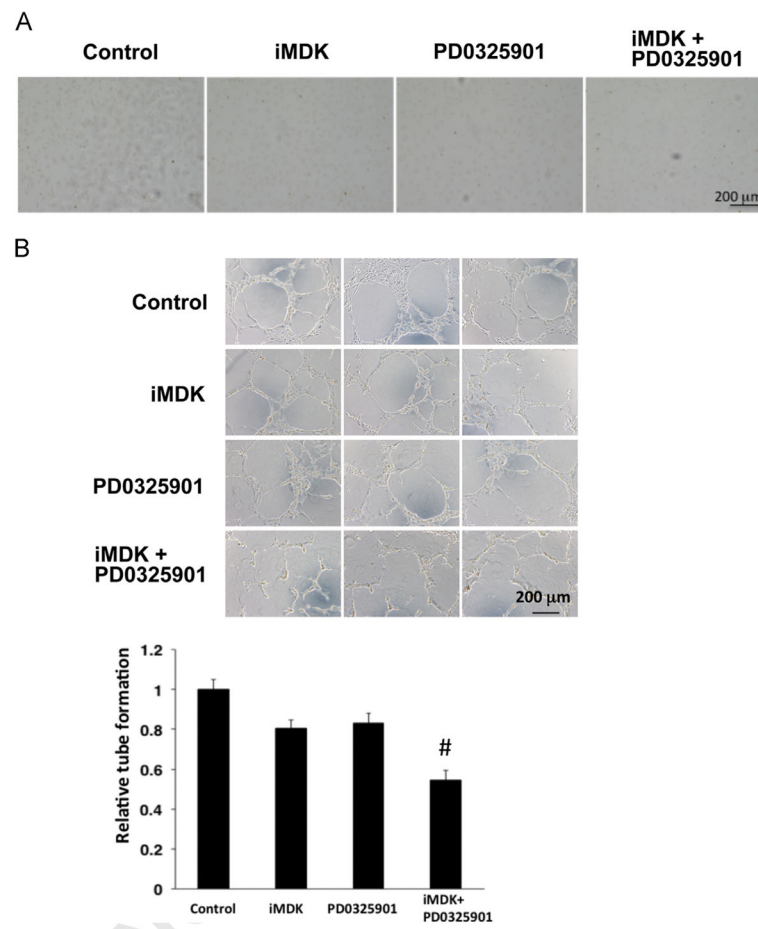


Fig. 4. iMDK suppressed angiogenesis with PD0325901 *in vitro*

A. Treatment of HUVEC cells with iMDK, PD0325901 or a combination of the two did not induce cell death as detected by TUNEL staining. Cells were treated for 5 h at a concentration of 10 μM. TUNEL staining was performed as described in *Materials and methods*.

B. Combinatorial treatment of iMDK with PD0325901 significantly inhibited tube formation of HUVECs. HUVEC cells were treated with iMDK (10 μM), PD0325901 (10 μM) or both for 5 h. Tube length was quantified as detailed in *Materials and methods*. Statistical significance was defined as $p < 0.05$ (#).

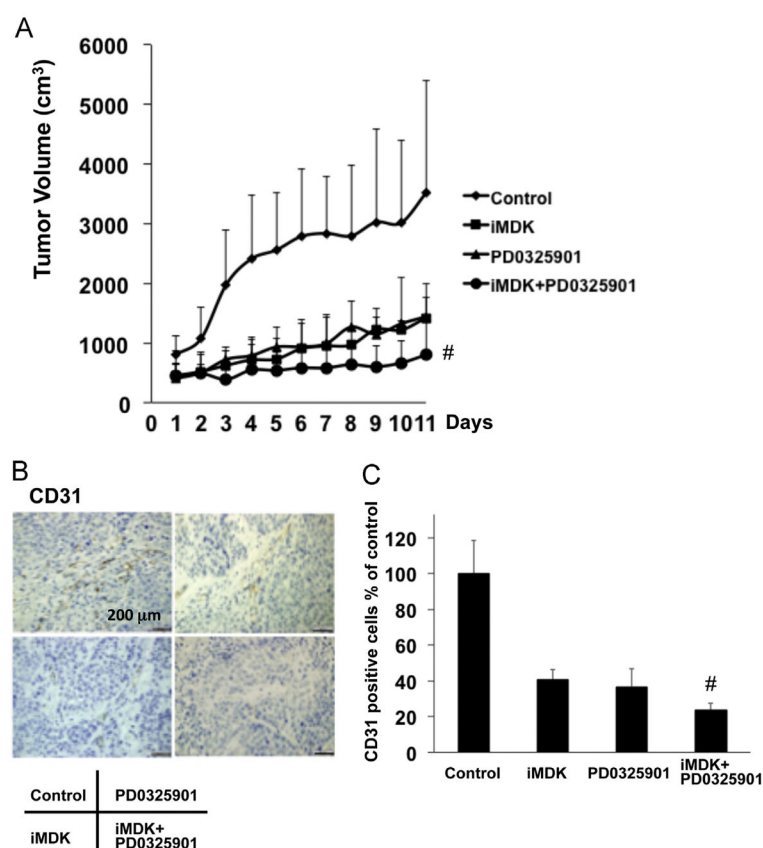


Fig. 5. The combination treatment of iMDK and PD0325901 effectively reduced lung tumor growth in a xenograft mouse model

A. Volume of the tumors derived from H441 lung adenocarcinoma cells was significantly reduced after the combination treatment with iMDK (9 mg/kg) and PD0325901 (5 mg/kg) compared to that of single compound in a xenograft mouse model. Eight mice were used in each group. Tumor volume was monitored every day after inoculation of H441 cells. Tumor growth is expressed as mean tumor volume; bars represent SD. Statistical significance was defined as $p < 0.05$ (#).

B. Shown is an image of xenograft tumors derived from H441 cells, which are dissected from the xenograft mice 11 days after treatment with iMDK. Expression of an angiogenesis marker CD31/PECAM-1 was detected by immunohistochemistry (scale bar shows 200 μ m). C. Expression of CD31/PECAM-1 was significantly inhibited by the combination treatment of iMDK and PD0325901 compared to that of single compound treatment. Statistical significance was defined as $p < 0.05$ (#).